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Relationship between the Metabolite Profile and Technological Properties of Bovine Milk from Two Dairy Breeds Elucidated by NMR-Based Metabolomics

Ulrik Kræmer Sundekilde,[†] Pernille Dorthea Frederiksen,[‡] Morten Rahr Clausen,[†] Lotte Bach Larsen,[‡] and Hanne Christine Bertram*[,†]

[†]Department of Food Science, Research Centre Aarslev, Aarhus University, Kirstinebjergvej 10, DK-5792 Aarslev, Denmark

[‡]Department of Food Science, Research Centre Foulum, Aarhus University, Blichers Alle 20, DK-8830 Tjele, Denmark

Supporting Information

ABSTRACT: The aim of the present study was to investigate the relationship between the metabolite profile of milk and important technological properties by using nuclear magnetic resonance (NMR)-based metabolomics. The metabolomics approach was introduced for the metabolic profiling of a set of milk samples from two dairy breeds representing a wide span in coagulation properties. The milk metabolite profiles obtained by proton and carbon NMR spectroscopy could be correlated to breed and, more interestingly, also with the coagulation profile, as established by traditional methods by using principal component analysis (PCA). The metabolites responsible for the separation into breed could mainly be ascribed to carnitine and lactose, whereas the metabolites varying in the samples with respect to coagulation properties included citrate, choline, carnitine, and lactose. The results found in the present study demonstrated a promising potential of NMR-based metabolomics for a rapid analysis and classification of milk samples, both of which are useful for the dairy industry.

KEYWORDS: dairy milk, coagulation properties, citrate, choline, nuclear magnetic resonance spectroscopy, lactose

INTRODUCTION

Milk is a complex biological fluid secreted for neonate nourishment and development. Basically, milk contains water, lipids, carbohydrates, proteins, vitamins, minerals, and smaller metabolites. It is well-known that the metabolite composition of bovine milk changes with stage of lactation, feed, seasonal changes, genetic variability, and also the health status of the cow.[1−6] Compositional changes affect both nutritional and technological properties of the milk. Accordingly, the concentration of lactose increases, whereas oligosaccharides decrease from colostrum to the later lactation stages,[6,7] and seasonal variations in both lipid and carbohydrate composition have been reported.[1,2] Furthermore, fish oil and sunflower oil addition in the diet specifically increases the concentration of the beneficial *cis*-9,*trans*-11 conjugated linoleic acid in the milk.[8]

In the dairy industry, the physical or technological properties of milk from dairy cows are of great importance, for example, properties associated with cheesemaking ability, such as rennet coagulation properties and syneresis of curd. Formation of a cheese curd is a two-step process; the first step is enzymatic hydrolyzation of κ-casein, producing para-κ-casein and hydrophilic macromolecules. The second step is syneresis, when the curd forms due to a reduction in colloidal stability of the micelles caused by the removal of the hydrophilic macromolecules.[9] The rennet coagulation properties of the milk are key aspects in cheese production, influencing cheese yield and quality, and include rennet coagulation time (RCT), curd firming rate (CFR), and curd firmness (G_{max}). Coagulation properties are influenced by a range of different factors such as molecular composition of the milk and also lactation stage, breed, feeding, and season.[1,2,4,10,11] The molecular composition includes the amount of casein, milk pH, and also the availability of the different ions present in milk, namely, calcium, phosphorus, sodium, and potassium.[12,13] Thus, both the nutritional and technological quality of the milk are closely associated with the composition of the milk and the level of different metabolites and micronutrients. Despite this fact, few studies have elucidated the relationship between the metabolite profile of milk and its technological properties. Furthermore, given the many factors controlling the cheesemaking properties, the ability to rapidly predict the technological properties of milk would be advantageous.

Metabolomics has become a widely applied approach for studying how various factors and/or stimuli cause perturbations in the overall composition of metabolites in a sample. A tremendous number of metabolomic studies have been reported on biofluid samples including urine, blood, and saliva, and nuclear magnetic resonance (NMR) has been proven to be an excellent analytical technique for the purpose.[14] There have only been a few attempts of using metabolomics in the study of milk, even though milk, by nature of its fluid state, should be an ideal sample for NMR-based metabolomics. However, the colloidal nature and restricted mobility of the different components in milk lead to a wide range of relaxation times and widths of resonance lines in NMR spectroscopy. The casein micelles and the fat globules, holding the majority of the protein and fat molecules in milk, are

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large aggregates with low mobility compared to smaller components, for example, lactose, choline, carnitine, and citrate. Thus, sample preparation presents a crucial step in obtaining high-quality data for NMR-based metabolomics from milk samples. Previously, various kinds of NMR spectroscopy have been used for the study of milk; among these, ^{31}P NMR spectroscopy has been used for studying caseins and whey proteins.[15] Recently, the use of proton NMR spectroscopy has been employed in the study of milk composition, showing the ability of proton NMR to detect and characterize more than 20 different metabolites including lactose, choline, carnitine, citrate, and creatinine.[3,16] Furthermore, proton NMR and two-dimensional HSQC NMR have been shown to be able to quantitate a number of compounds, including lipids, lactose, and citrate, in milk.[3,17] Consequently, it is hypothesized that NMR-based metabolomics is a useful technique for elucidating the impact of the milk metabolite profile on technological properties of the milk, and the aim of the present study is to explore the potential of NMR-based metabolomics in the prediction of functional properties of milk by using a set of bovine milk samples previously characterized by their coagulation properties and known to represent both poor and good coagulating milk from two different breeds.[18]

MATERIALS AND METHODS

Samples and Sample Collection. A total of 14 individual milk samples from the two dairy breeds, Danish Jersey (JER) and Danish Holstein-Friesian (SDM), were collected as previously described.[18] Cows were housed in a loose housing system with a free cow traffic system, milked by an automatic and voluntary milking system (DeLaval, Tumba, Sweden), and fed ad libitum with a total mixed ratio and supplemented with concentrate according to standard practice. Information regarding daily milk yields, milk yield in the actual milking, parity, lactation stage, and time since last milking was registered for each milk sample collected.[18] The selected cows and samples were part of a larger screening study of variations in coagulation properties of milk from different Danish dairy breeds and included 53 SDM and 38 Jersey cows.[18] On the basis of the milk coagulation properties of milk samples taken on an individual basis, 10 cows producing good coagulating milk and 10 cows producing poorly coagulating milk from each of these two breeds were followed during a 7 month follow-up period, and 7 cows consistently producing poorly coagulating milk (4 SDM and 3 Jersey) and 7 cows producing good coagulating milk (4 SDM and 3 Jersey) were selected for comparison. From each cow a 250 mL sample of milk was manually collected from one milking event. The milk samples were immediately cooled and refrigerated at 5 °C for 1−4 h without preservatives before preparation for rheological analyses. Furthermore, aliquots of skimmed milk samples were frozen at −80 °C prior to the NMR spectroscopy.

Rheological Analyses and Coagulation Properties. Each milk sample was analyzed for concentration of milk fat, protein, casein, and lactose by a Milkoscan FT 6000 (Foss Electric, Hillerød, Denmark) and for somatic cell count using a Fossomatic 5000 (Foss Electric) at Eurofins Laboratory (Holstebro, Denmark). All milk samples included in the study had somatic cell counts below 500×10^3 cells/mL. Measurements of the coagulation properties (RCT, CFR, and G_{max}) were performed by continuous measurement during renneting using a ReoRox4 rheometer (Medirox, Nyköping, Sweden) as previously described.[18] Essentially, 10 mL of milk was skimmed by centrifugation at 200*g* for 20 min at 4 °C and adjusted to pH 6.5 with 10% (v/v) lactic acid. The milk sample was then preincubated for 30 min in a water bath at 33 °C before the addition of 0.038 IMCU/mL milk of ChyMax Ultra (Christian Hansen Laboratories A/S, Hørsholm, Denmark). A good/poor composite coagulation score was created on the basis of the information given by RCT, CFR, and G_{max}.

NMR Spectroscopy. Prior to NMR spectroscopy the skimmed milk samples were thawed and thoroughly shaken to homogenize the sample. Four hundred microliters of sample was added to 200 μL of D_2O containing sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate (TSP; Sigma-Aldrich, Brøndby, Denmark) as an internal chemical shift reference. Four replicates of each sample were prepared for ^{1}H NMR spectroscopy, whereas for ^{13}C NMR spectroscopy each sample was measured only once due to longer measurement time. ^{1}H NMR spectroscopy was performed at 298 K on a Bruker Avance III 600 spectrometer, operating at a ^{1}H frequency of 600.13 MHz, and equipped with a 5 mm ^{1}H TXI probe (Bruker BioSpin, Rheinstetten, Germany). Standard one-dimensional spectra were acquired using a single 90° pulse experiment with a relaxation delay of 5 s. Water suppression was achieved by irradiating the water peak during the relaxation delay, and a total of 32 scans were collected into 32K data points spanning a spectral width of 12.15 ppm. In addition ^{13}C NMR spectroscopy was performed at 298 K on a Bruker Avance III 800 spectrometer operating at a ^{13}C frequency of 200.98 MHz and equipped with a 5 mm TCI cryoprobe optimized for ^{1}H and ^{13}C observation (Bruker BioSpin). Proton-decoupled ^{13}C NMR spectra were recorded using Bruker standard pulse programs, and each spectrum was the sum of 1024 free induction decays. A 90° pulse was applied with a repetition time of 2 s and an acquisition time of 0.599 s. NMR signals have been assigned in accordance with existing literature.[3,16]

All ^{1}H and ^{13}C spectra were initially referenced to the TSP signal at 0 ppm. Prior to Fourier transformation the data were multiplied by a 0.3 Hz line-broadening function. The proton NMR spectra were phase- and baseline-corrected manually, and ^{13}C spectra were phase-corrected manually and baseline-corrected by cubic spline correction using Topspin 2.1 (Bruker BioSpin).

Multivariate Data Analysis. The ^{1}H NMR spectra were aligned using Icoshift by coshifting of the whole spectra according to the lactose doublet at 4.68 ppm.[19] The ^{1}H NMR spectra were acquired at the same instrument settings; thus, no normalization is required. The proton NMR spectra were subdivided into 0.02 ppm integral regions and integrated, reducing each spectrum into 362 separate variables in the regions 10.00−5.00 and 4.70−2.46 ppm. The ^{13}C spectra were aligned using Icoshift[19] by coshifting of the whole spectra according to TSP. Furthermore, the ^{13}C NMR spectra were normalized to the externally measured lactose concentration due to differences in spectrometer settings (receiver gain). Finally, the spectra were subdivided into 0.04 ppm integral regions and integrated, reducing each spectrum into 4099 separate variables in the region 181−0.5 ppm. Prior to multivariate data analysis, different scaling methods of the NMR variables were tested, including Pareto scaling and scaling to unit variance. Principal component analysis (PCA) was then applied to the centered data to explore any clustering behavior of the samples separately in proton and carbon NMR. All PCA models were validated using segmented cross-validation, wherein each segment represents the replicates of each sample. Q^2 is a measure of this cross-validation, and the values are indicated in the figure captions. Multivariate data analysis was performed using SIMCA-P+ 12.0.1.0 (Umetrics AB, Sweden). Alignment using Icoshift and binning were performed in MATLAB 7.9 (MathWorks Inc. USA).

RESULTS AND DISCUSSION

NMR Metabolite Profiling of Milk Samples. NMR-based metabolomics has emerged as a widely applied approach in many areas of life sciences. However, the use of NMR-based metabolomics for elucidating food quality is still in its early phase. Recently, NMR-based metabolomics have been successfully applied to study variations in the milk metabolite profile during lactation,[3] but otherwise metabolomic investigations on milk are sparse. One drawback of using NMR to study the metabolite variability of various biofluids is the lower sensitivity compared to

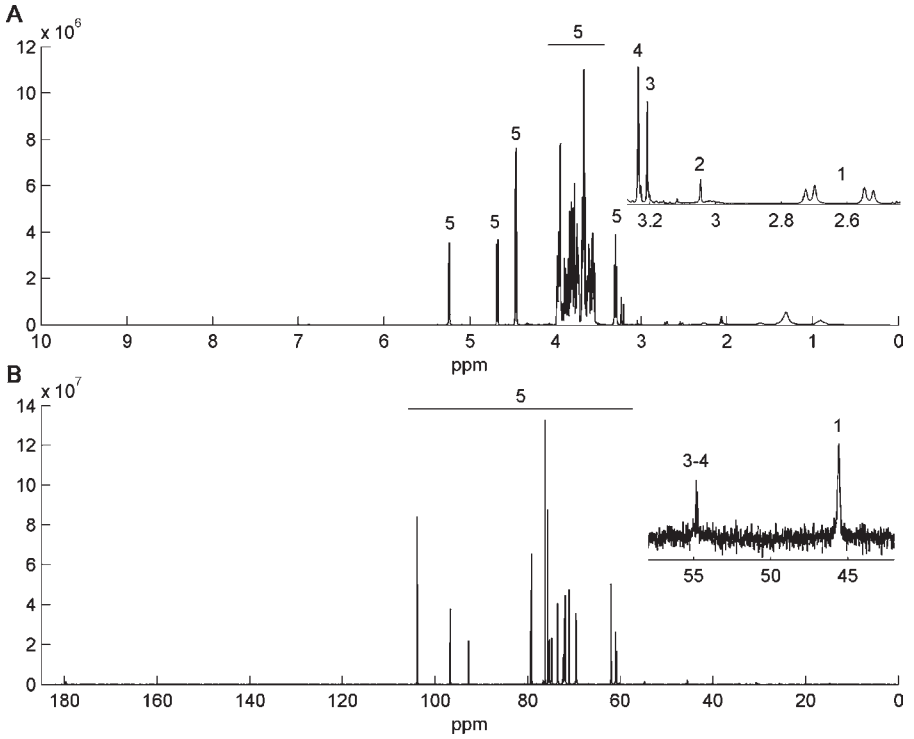

Figure 1. (A) Representative ^{1}H NMR spectrum obtained from a single skimmed milk sample. (B) Representative ^{13}C NMR spectrum obtained on a milk sample. Assignment of NMR resonances: 1, CH_2 in citrate; 2, CH_3 in creatinine; 3, CH_3 in choline; 4, CH_3 in carnitine; 5, various resonances from protons in lactose.

other methods such as mass spectrometry. Recently, the biochemical variability of bovine milk was studied using MS, enabling the characterization of over 200 different metabolites.[20] However, compared with MS-based high-throughput methods, NMR spectroscopy can also be considered a high-throughput method with the advantages of minimal sample preparation and high reproducibility.[21]

In the present study, high-resolution ^{1}H and ^{13}C NMR spectra were obtained on bovine skimmed milk samples and application of metabolomics was applied to investigate differences between the samples. Figure 1A shows a representative high-resolution ^{1}H NMR spectrum obtained on a skimmed bovine milk sample. Several signals from lactose (5), other carbohydrates, and low molecular weight metabolites, for example, carnitine (4), choline (3), creatinine (2), and citrate (1), were detected. Figure 1B shows a representative high-resolution ^{13}C NMR spectrum obtained for a skimmed bovine milk sample. Multiple signals in the ^{13}C spectrum can be assigned to lactose (5); however, also signals from lipids, citrate, choline, taurine, and carnitine are present. The signals were assigned by comparison with chemical shifts reported in the literature and the Human Metabolome Database[3,16,22] and by use of a two-dimensional ^{1}H−^{13}C heteronuclear single quantum coherence (HSQC) spectrum of some of the milk samples (data not shown). Some of these assignments are listed in the figure captions, and numbers in parentheses refer to numbering of peaks in Figure 1. In principle, ^{1}H NMR spectroscopy detects protons in smaller, mobile metabolites, and because the concentration of lactose is high in milk, signals from lactose are expected to have substantial impact on the metabolite profile obtained by ^{1}H NMR spectroscopy. Thus, comparison of ^{1}H NMR spectra obtained on bovine milk samples with a ^{1}H NMR spectrum of a pure lactose solution revealed great resemblance (data not shown). Furthermore, NMR signals from citrate, choline, carnitine, and creatinine were also assigned. As mentioned, the sample preparation step is crucial in obtaining high-quality data. ^{1}H NMR spectroscopy on whole milk is largely obscured by large resonances stemming from lipid acyl chains; thus, we decided on analyzing skimmed milk samples. Furthermore, the rheological analyses and assessment of coagulation properties were performed on skimmed milk. Thus, correlations between the rheological analyses and the NMR profile should be feasible. Figure 1A shows the full ^{1}H NMR spectrum of a skimmed milk sample; the residual lipid (remnants from the skimming process) acyl chain resonances are visible between 2.4 and 0.5 ppm (Figure 1A). As these resonances vary with the skimming process, we have discarded them in the following multivariate data analysis. As for the ^{1}H NMR spectra, the ^{13}C NMR spectra were also dominated by signals corresponding to lactose, along with less intensive signals from residual lipids and from taurine, citrate, choline, and carnitine (Figure 1B).

Classification of Milk Samples into Breeds by NMR Metabolite Profiling. The milk samples included in the present study originated from two different dairy breeds, and PCA was applied to the ^{1}H and ^{13}C NMR data to identify possible variations in the metabolite composition between the different samples and possibly breeds. Multiple PCA models were tested for classification of milk samples into breeds, testing both variable bin sizes and different scalings of the data. The Pareto scaled model shows the same groupings, but does not improve the classification (data not shown). Thus, the PCA model used for classification is built on the mean-centered ^{1}H NMR data, including four replicates of each sample, and it demonstrates different clustering patterns. Figure 2A shows that by ^{1}H NMR milk samples can be grouped according to breed by principal components 1 (PC1) and 4

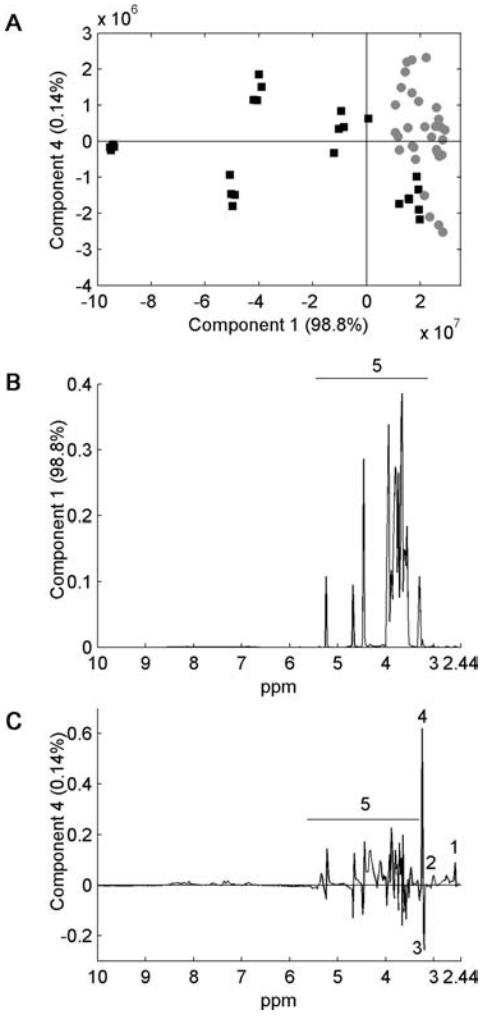

Figure 2. (A) PCA score plot of ^{1}H NMR data on skimmed milk showing principal components 1 and 4 colored according to Jersey (black squares) and Holstein (gray circles) breeds. (B, C) Corresponding loading plots of (B) PC1, Q^2 value of PC1 = 0.981; and (C) PC4, Q^2 value of PC4 = 0.122. Numbers refer to assignments of resonances in Figure 1.

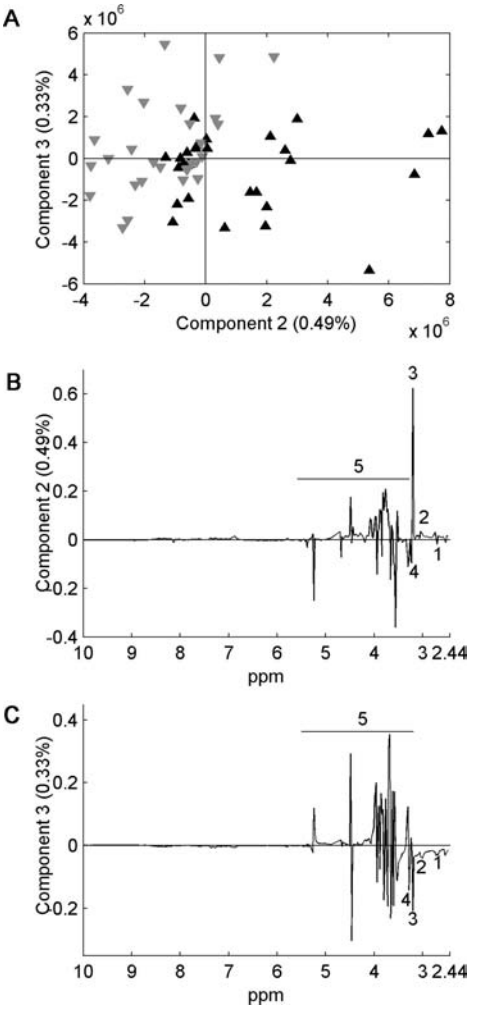

Figure 3. (A) PCA score plot of ^{1}H NMR data showing principal components 2 and 3 colored according to good (black triangles) and poor (gray triangles) coagulation properties. (B, C) Corresponding loading plots; (B) PC2, Q^2 value of PC2 = 0.09; and (C) PC3, Q^2 value of PC3 = 0.395. Numbers refer to assignments of resonances in Figure 1.

(PC4), explaining 98.8 and 0.14% of the variance, respectively. Analysis of the loadings for the present PCA model reveals variables of importance for the classification into breeds, and by displaying the loadings as a line plot, which resembles the original spectrum, the important variables are revealed. PC1 highly resembles the original spectrum, which is expected as the most dominant protons measured by ^{1}H NMR are present in lactose (Figure 2B), as also concluded from Figure 1. Thus, PC1 is characterizing lactose. Indeed, there are differences in lactose concentration among the milk samples analyzed, which were also found by FTIR.[18] Lactose concentration ranged from 2.27 to 4.82 g × 100/g.[18] PC4 is also involved in the discriminative ability of the model to classify samples according to breed. PC4 explains 0.14% of the variance; thus, it explains hypothetically minute differences between the samples. However, because of the large dynamic range between lactose and less abundant metabolites such as citrate, carnitine, and choline, together with the fact that the variables are not scaled, the explained variance is relative to the differences in lactose resonances. Analysis of the loading line plot of PC4 shows that signals at 3.20 ppm (3) and 3.24 ppm (4), and to a smaller extent signals at 2.56 ppm, 2.74 ppm (1), and 3.0 ppm (2), and the multiple signals from lactose at 3.3−5.24 ppm (5), influence the grouping (Figure 2B). The peak at 3.20 ppm is assigned to CH_3 protons in choline, and the 3.24 ppm peak is assigned to CH_3 protons in carnitine. Peaks at 2.56 and 2.74 ppm represent CH_2 protons in citrate, and the peak at 3.0 ppm represents CH_3 protons in creatinine. Peaks in the interval 3.5−4.0 ppm and the doublets at 4.46, 4.68, and 5.24 ppm represent various protons originating from lactose. Positive peaks in PC1 indicate that these metabolites are present in higher levels in milk of SDM origin as these milk samples all are located in the positive area of PC1 in the score scatter plot (Figure 2A,B). The concentrations of carnitine and citrate are relatively higher in milk from Jersey cows than in milk from SDM cows as seen from the ^{1}H NMR metabolite profile, whereas choline concentration is relatively higher in milk from SDM cows than in milk from Jersey cows (Figure 2).

Classification of Milk Samples According to Coagulation Properties. The milk samples analyzed in the present study are known to express a variation in technological quality ranging

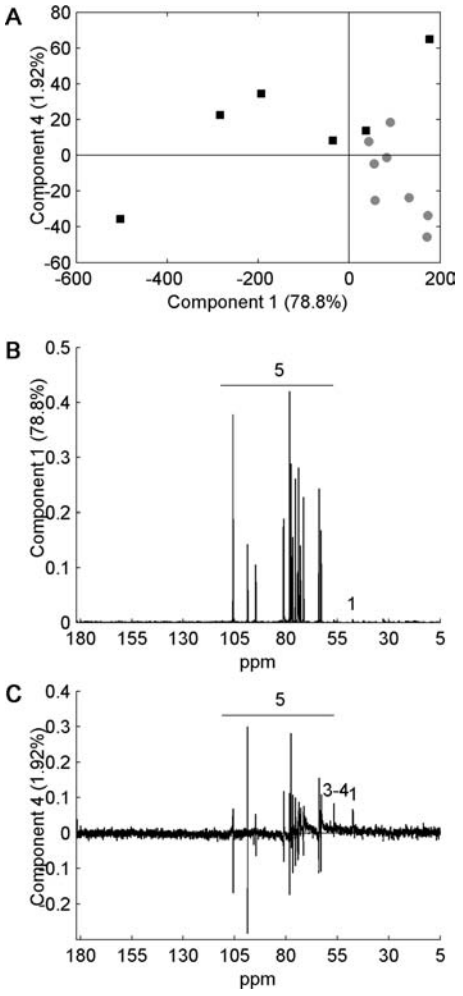

Figure 4. (A) PCA score plot of ^{13}C NMR data on skimmed milk showing principal components 1 and 4 colored according to Jersey (black squares) and Holstein (gray circles) breeds. (B, C) Corresponding loading plots of (B) PC1, Q^2 value of PC1 = 0.745; and (C) PC4, Q^2 value of PC4 = 0.004. Numbers refer to assignments of resonances in Figure 1.

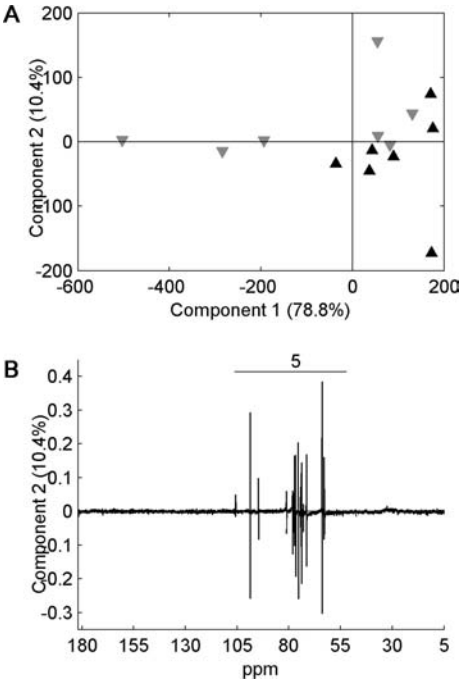

Figure 5. (A) PCA score plot of ^{13}C NMR data on skimmed milk showing the two first principal components colored according to good (black triangles) and poor (gray triangles) coagulation properties. (B) Corresponding loading plot of (B) PC2, Q^2 value of PC2 = 0.34. PC1 is shown in Figure 4B. Numbers refer to assignments of resonances in Figure 1.

from noncoagulating milk to milk that quickly coagulates and aggregates, thereby forming a solid gel. A high aggregation rate and a firm coagulum are essential to obtain a low loss of fines to the whey during the cheesemaking process, thereby optimizing the cheese yield.[23] Consequently, the milk samples have been scored into a composite coagulation score describing how well the milk coagulates. The composite coagulation score was determined according to the coagulation properties of each milk sample measured by free oscillation rheometry. Good-coagulating milk had low to medium RCT, high CFR, and high G_{max} (RCT $\leq$ 10 min, CFR $\geq$ 42 Reorox units, and $G_{max} \geq$ 623 Reorox units), whereas poor-coagulating milk was characterized by medium to high RCT, low CFR, and low to medium G_{max} (RCT $\geq$ 10 min, CFR $\leq$ 35 Reorox units, and $G_{max} \leq$ 519 Reorox units).[18] Further details on the milk composition are given in ref 18. Using ^{1}H NMR data, a PCA score scatter plot of the second and third principal components, which explain 0.49 and 0.33% of the variance, respectively, is able to group the milk samples according to coagulation properties (Figure 3A). Analysis of the corresponding loading plots shows that choline (3 and an additional peak at 4.06 ppm) is the most dominating metabolite responsible for the observed grouping along PC2. Choline is present in higher concentration in good-coagulating milk samples compared to poor-coagulating milk samples (Figure 3B). NMR signals originating from protons in lactose (5) at 3.5−4.0, 4.46, 4.68, and 5.24 ppm also have influence on the grouping according to coagulation properties (Figure 3B). A model based on Jersey milk samples is able to classify the samples according to their coagulation properties exclusively by ^{1}H NMR signals of lactose. Indeed, the poor-coagulating Jersey samples all had low lactose concentration compared to the good-coagulating samples (data not shown). Whereas lactose per se is not directly involved in the coagulation processes, it can be an indicator for a changed metabolite composition of the milk. Given that 98.8% of the variance is explaining lactose, variable selection is applied to highlight metabolites other than lactose. Thus, a model in which all signals from lactose are removed shows a better discriminative ability than the original model with PC1 and PC5 (52.8 and 3.68% explained variance, respectively). The corresponding loading plots show that choline and carnitine are dominating the grouping along PC1, whereas citrate, along with choline and carnitine, is dominating along PC5 (Supporting Information). There is a tendency of higher citrate concentration in samples with poor coagulation properties (Figure 3B and Supporting Information). Furthermore, choline concentration is higher and carnitine concentration lower in good-coagulating milk samples, thereby emphasizing the results found in the original model (Supporting Information).

Score plots obtained from PCA on the mean-centered ^{13}C NMR data demonstrate similar clusters as observed on ^{1}H NMR data (Figures 4 and 5). The score scatter plot of PC1 and PC4 groups the samples into breeds, thereby indicating differences in milk composition between milk samples from Jersey and SDM

breeds (Figure 4A). PC1 explains 78.8% of the variance in the data set, whereas PC4 explains 1.92% of the variance in the data set. The loading plot of the first component shows high similarity with the original ^{13}C NMR spectrum, indicating that signals from lactose dominate the clustering behavior (Figure 4B). PC4 also shows influence of lactose as well as citrate and choline/carnitine (Figure 4C). Furthermore, the PCA model built on ^{13}C NMR data is able to discriminate between the coagulation properties of the milk. A PCA model of the first two principal components, explaining 78.8 and 10.4% of the variance, respectively, is shown in Figure 5A. As mentioned, the loading plot of PC1 mainly shows lactose, indicating that signals from lactose dominate the clustering behavior (Figure 4B), whereas PC2 also explains inter-lactose differences (Figure 5B). Thus, the coagulation properties are reflected exclusively by variability of lactose resonances in ^{13}C NMR spectroscopy.

The coagulation properties of milk are important for the dairy industry. In the present study, we demonstrate that information about coagulation properties can be extracted from the NMR metabolite profiles of skimmed milk. The metabolites responsible for the prediction of coagulation properties include choline, carnitine, citrate, and, to some degree, also lactose. The positive effect of lactose concentration on the coagulation properties found in the present study is in agreement with the fact that an increasing concentration of lactose in milk has been shown to exert a beneficial effect on the formation of acid-induced milk gels.[11] Furthermore, lactose is the most important osmoregulator of milk synthesis and is to some extent correlated with the total protein and casein content of milk, thereby positively influencing the coagulation properties, of which especially the CFR and G_{max} values are influenced by the total protein content and the casein content.[24]

In the PCA model in which variable selection is applied (Supporting Information), there is a larger influence of citrate along the fifth principal component than experienced in the PCA model displayed in Figure 3A. There is a tendency toward higher concentrations of citrate in samples with poor coagulation properties. In bovine milk, citrate, which is associated with the whey fraction, exists either in complexes with calcium and magnesium (85%) or in free forms (14% as citrate^{3-} and 1% as hydrogen citrate^{2-}).[9] Consequently, citrate acts as a calcium chelating agent in milk, thereby solubilizing the colloidal calcium phosphate, and disrupts the casein micelle structure affecting coagulation parameters.[13,18] As the calcium−citrate complex has a greater stability constant than that of calcium−phosphate, increased levels of citrate in the whey will affect the level of colloidal (micellar bound) calcium phosphate and thereby the coagulation properties,[9] and a correlation between soluble calcium and citrate has earlier been observed.[25] The present findings are in agreement with the fact that addition of citrate reduces the storage modulus (G') of rennet-induced gels, and above a certain concentration of citrate, rennet coagulation is completely inhibited.[13] Our findings further substantiate the results of Udabage et al.,[13] who demonstrated that also naturally occurring variations in citrate concentration in the milk affect the coagulation properties. A tendency toward higher citrate content in poor-coagulating milk in comparison with good-coagulating milk has recently been demonstrated.[18] Furthermore, citrate levels vary with stage of lactation and are influenced by the cow's diet as it is an intermediate in the de novo fatty acid synthesis.[26]

As mentioned, the present study includes a subset of samples from a larger sample set, and as expected there is a clear effect of the lactose concentration in discriminating the two breeds, which is in agreement with the fact that the lactose concentration was found to be significantly different between SDM and Jersey breeds.[18] One of the advantages of using chemometrics as a data mining tool is the ability of seeing structures beyond the dominating signals. By using an explorative approach, the present study reveals that in addition to lactose, a combination of signals arising from choline, carnitine, and citrate is the major difference in the milk metabolite profile between the two breeds (Figure 2). The ^{1}H NMR and ^{13}C NMR metabolite profiles show differences in the relative abundances of carnitine, choline, and citrate in milk samples from the two breeds. The concentrations of carnitine and citrate are relatively higher in milk from Jersey cows than in milk from SDM cows, and choline concentration is relatively higher in SDM milk than in Jersey milk. A number of systematic differences between breeds have previously been found.[3] Choline is essential and ubiquitous in the diet[27] and is of high nutritional value, as it is abundant in cell membranes and acts as a precursor for the important messenger acetylcholine. Accordingly, choline is crucial during the early development of off-spring, and it is important as well for the adult in preventing liver and muscle damage and other health risks.[28] Carnitine is an important metabolite, seen from a nutritional point of view, as it is active in the transport of long-chain fatty acids from the cytosol to the mitochondrial matrix. Opposite from choline, carnitine is seen as only conditionally essential; accordingly, both premature and formula-fed infants need a source of carnitine in their diet.[29,30] Carnitine deficiency has been shown to have wide systemic effects on the infant, possibly leading to death, highlighting its importance.[31] During infancy, milk and infant formula derived from milk are often the sole source of nutrition; thus, the concentrations of these metabolites in bovine milk are important from a nutritional perspective. Hence, milk with a higher content of these metabolites could be advantageous. As discussed above, the importance of choline and carnitine in nutrition is well established, whereas there has not been much work done elucidating the effects of choline and carnitine on milk coagulation properties. The explorative approach in the present study demonstrates a possible relationship between choline and carnitine and the technological quality of the milk. It is important to note that the choline and carnitine that are visible by proton NMR are free choline and carnitine. The discriminative ability of choline and carnitine in differentiating poor/good-coagulating milk samples could very well be indirect, and further studies are needed to understand the relationship between choline and carnitine and coagulation properties of the milk in regard to cheesemaking processes.

In conclusion, by using NMR-based metabolomics, the present study demonstrated a clear relationship between the metabolite profile and the coagulation properties of bovine milk. The multivariate data analyses revealed that the NMR data could discriminate between milk samples with poor and good coagulation properties. To the authors' knowledge, the present study is the first to demonstrate a relationship between the NMR metabolite profile of milk and its technological properties. Breed information could also be extracted from the NMR metabolite profile of milk. The nontargeted approach revealed a negative relationship between citrate in milk and the coagulation properties. Furthermore, the levels of lactose, carnitine, and choline in the milk were also found to be associated with the coagulation properties. Consequently, our results have unraveled new information about milk metabolites that are of importance for the coagulation properties. Further studies elucidating the exact mechanisms by which these metabolites influence coagulation properties could be of great interest.

■ ASSOCIATED CONTENT

Supporting Information. Supporting Information Figure 1: (A) PCA score plot of partial ^{1}H NMR data showing principal components 1 and 5 colored according to good (black triangles) and poor (gray triangles) coagulation properties. The ^{1}H NMR data have been subjected to variable selection, and resonance signals from protons in lactose have been excluded. (B, C) Corresponding loading plots of (B) PC1 and (C) PC5. Numbers refer to assignments of resonances in Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (+45) 8999 3344. Fax: (+45) 8999 3494. E-mail: HanneC.Bertram@agrsci.dk.

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